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Biophysical characterization of actin bundles generated by the *Chlamydia trachomatis* Tarp effector

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ABSTRACT

Chlamydia trachomatis entry into host cells is mediated by pathogen-directed remodeling of the actin cytoskeleton. The chlamydial type III secreted effector, translocated actin recruiting phosphoprotein (Tarp), has been implicated in the recruitment of actin to the site of internalization. Tarp harbors G-actin binding and proline rich domains required for Tarp-mediated actin nucleation as well as unique F-actin binding domains implicated in the formation of actin bundles. Little is known about the mechanical properties of actin bundles generated by Tarp or the mechanism by which Tarp mediates actin bundle formation. In order to characterize the actin bundles and elucidate the role of different Tarp domains in the bundling process, purified Tarp effectors and Tarp truncation mutants were analyzed using Total Internal Reflection Fluorescence (TIRF) microscopy. Our data indicate that Tarp mediated actin bundling is independent of actin nucleation and the F-actin binding domains are sufficient to bundle actin filaments. Additionally, Tarp-mediated actin bundles demonstrate distinct bending stiffness compared to those crosslinked by the well characterized actin bundling proteins fascin and alpha-actinin, suggesting Tarp may employ a novel actin bundling strategy. The capacity of the Tarp effector to generate novel actin bundles likely contributes to chlamydia's efficient mechanism of entry into human cells.

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1. Introduction

The obligate intracellular bacterium, *Chlamydia trachomatis* is the most frequently reported sexually transmitted bacterial disease in the United States, with over 1 million cases reported annually to the Centers for Disease Control and Prevention (CDC) since 2006 [1]. *C. trachomatis* displays a unique biphasic developmental cycle consisting of two metabolically and morphologically distinct developmental forms [2]. The infectious extracellular form is called the elementary body (EB) whereas the vegetative intracellular form is called the reticulate body (RB) [3].

To facilitate the obligate intracellular lifestyle, *Chlamydia trachomatis* manipulates the host cell cytoskeleton to promote entry, development and exit [4]. Shortly after attachment of the EB to the host cell surface, *C. trachomatis* delivers several effector proteins into the host cell cytoplasm via a type III secretion system (T3SS) [5]. The translocated actin-recruiting phosphoprotein (Tarp) is one of the early translocated effectors and is spatially and temporally associated with the recruitment of actin to the site of EB invasion [6]. Tarp is a bacterial actin nucleating and bundling protein which harbors one G-actin binding domain (implicated in actin nucleation) as well as two F-actin binding domains (implicated in actin bundling) [7,8].

The arrangement of actin filaments during entry of the EBs into the host cell is not known. One of the well characterized actin bundling proteins, fascin 1, co-localizes with filopodia on the leading edge of the growth cones of developing nerve cells and are implicated in the formation of actin bundles [9]. Likewise, Tarp may play a role in the creation of actin bundles located directly beneath the host-pathogen contact site to form pedestal-like structures that are important for chlamydial entry into host cells [8,10]. Herein, we

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examined the biophysical properties of Tarp-generated actin bundles *in vitro* and thus demonstrate that Tarp-mediated actin bundle assembly is independent of actin nucleation and the F-actin binding domains are sufficient to bundle actin filaments. Additionally, Tarp-mediated actin bundles have distinct bending stiffness compared to that of known actin bundling proteins. To our knowledge, this is the first characterization of actin bundle flexibility engendered from a bacterial effector protein. Our findings indicate that Tarp employs a novel actin bundling strategy which may facilitate chlamydial invasion of human cells.

2. Materials and methods

2.1. Cloning, protein expression and purification

In-frame amino-terminal glutathione S-transferase (GST) and carboxyl-terminal polyhistidine fusion Tarp proteins were generated as previously described [8]. Two additional truncated Tarp effectors including the C-terminal domain of Tarp harboring the F-actin binding domain (FAB domain) (D^{761}-G^{1005}) and the N-terminal and central domains of Tarp excluding all known actin binding sites (N-terminal domain)(M^{1}-P^{747}) were generated by PCR amplifying the corresponding coding regions from *C. trachomatis* serovar L2 LGV 434 genomic DNA (Qiagen genomic purification kit, Valencia CA). PCR was performed with custom synthesized oligonucleotide primers (Integrated DNA technologies, Coralville, IA) engineered with BamHI and XhoI linkers. PCR products were purified, digested with restriction enzymes (New England Biolabs, Beverly, MA) and cloned into linearized pGEX-6P-1 vector (GE Health Sciences, Piscataway, NY) to generate the translation fusions. All clones were confirmed by restriction digest and Sanger sequencing. All Tarp containing pGEX-6P-1 plasmids were transformed into the BL21 strain of *Escherichia coli* (Novagen, Madison, WI). Protein expression and purification were performed according to the procedures outlined for Ni sepharose 6 Fast Flow and glutathione sepharose 4B in the bulk GST purification module (GE Health sciences, Chicago, IL). The GST tag was removed with PreScission Protease treatment according to the manufacturer's recommendations (GE Health Sciences, Chicago, IL).

2.2. Actin nucleation pyrene assay

Pyrene actin polymerization assays were performed as previously described [7,8,11].

2.3. F-actin binding and bundling assay

Actin monomers (21 μM) were first polymerized to form filamentous actin (F-actin) in the presence of polymerization buffer (10 mM imidazole, pH 7.0, 50 mM KCl, 2 mM $MgCl_2$) for 1 h at 25 °C. To induce bundles, F-actin was then incubated with 35 nM Tarp proteins for one more hour at 25 °C and spun at 10,000 × g for 30 min at 25 °C in a Beckman Optima TLX Ultracentrifuge using a TLA 100.3 rotor (Beckman Coulter Inc., Fullerton, CA). The low-speed centrifugation was used to separate bundles from F- or G-actin in samples. α-actinin (16 μM, Cytoskeleton, Denver, CO) was used as a positive control. For the one step polymerization/bundling assay, 500 nM of G-actin was incubated with increasing concentrations of wild type Tarp (0 nM–35 nM) for 2 h in presence of polymerization buffer at 25 °C and spun at 10,000 × g for 30 min at 25 °C. Equal volumes of supernatant and pellet were separated by 10% SDS-PAGE, stained with Coomassie blue for 1 h and destained overnight. Gels were analyzed by densitometry on a ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA).

2.4. TIRF microscopy imaging and bending persistence length analysis

Rhodamine-labeled G-actin (Cytoskeleton, Denver, CO) was polymerized in polymerization buffer (10 mM Imidazole, pH 7.0, 50 mM KCl, 2 mM $MgCl_2$, 1 mM ATP and 1 mM DTT) at the concentration of 8.3 μM for 1 h at room temperature (~22 °C) to form F-actin. Then the F-actin was incubated with Tarp, α-actinin (Cytoskeleton, Denver, CO) or fascin (Abcam, Cambridge, MA) at various molar ratio for 1 h at room temperature. Tarp-induced actin bundles were diluted with imaging buffer (10 mM imidazole, pH 7.0, 50 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 0.15 M glucose, 1 mg/ml catalase, 0.2 mg/ml glucose oxidase). Bundles were immobilized on poly-L-lysine (Sigma Aldrich, St. Louis, MO) coated microscope coverslips that were thoroughly cleaned with absolute ethanol and KOH followed by rinsing with ddH_2O. Bundle images were taken using a Nikon Eclipse Ti TIRF microscope equipped with a Hamamatsu Image EM X2 CCD Camera, a 100X oil immersion objective (numerical aperture, 1.49), and Nikon LU-N4 Laser. At least 100 filaments and/or bundles were analyzed for each sample. Actin filament and/or bundle length and bending persistence length (L_p) were calculated from the two-dimensional average cosine correlation ($\langle C(s) \rangle$) of the tangent angle (θ) along the segment lengths (s) of a filament and/or bundle by fitting to the following equation (where A is a scaling factor) as described [12] using ImageJ (NIH) and *Persistence* software:

$$< C(s) > \; = \; < cos[\theta(s) - \theta(0)] > \; = A * e^{-x/2L_p} \qquad (1)$$

2.5. Statistical analysis

Statistical analyses were performed using Graphpad Prism (version 7.04, Graphpad software, CA). Unless otherwise stated, data are given as mean ± 95% confidence intervals. Comparison between two groups were performed using Mann-Whitney *t*-test in case of non-parametric data and for multiple groups one-way analysis of variance (ANOVA) and Tukey's post hoc comparisons were used.

3. Results

3.1. The Tarp FAB domain is sufficient for actin bundle formation

C. trachomatis Tarp containing one G-**a**ctin **b**inding **d**omain (ABD) and two **f**ilamentous **a**ctin **b**inding domains (FAB) binds directly to both globular actin and filamentous actin *in vitro* respectively [8]. The ABD and **p**roline **r**ich **d**omain (PRD) are required for Tarp-mediated actin nucleation [7]. However, the cohort of protein domains required for Tarp-mediated actin bundling has not been thoroughly examined. In order to determine which region(s) of Tarp is sufficient for bundling actin filaments we generated recombinant wild type Tarp and mutant Tarp proteins that harbor specific domain deletions (Fig. 1A). Specifically, amino- and carboxyl-domain deletions were generated to create truncated Tarp proteins referred to as the FAB domain (deletion of amino acids M1 through P747) and the N-terminal domain (deletion of amino acids A748-G1005), respectively. A Tarp effector lacking the solitary G-actin binding domain (ΔABD) was also created (deletion of A748-K758) (Fig. 1A). The purified proteins (Fig. 1B) were analyzed for actin nucleation activity in pyrene actin polymerization assays (Fig. 1C). In agreement with previous reports [8], all mutant Tarp proteins lacking the G-actin binding domain (ΔABD, FAB domain and N-terminal domain) failed to promote actin

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